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LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein

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Abstract

It has been previously documented that the thermolysin-digest of "Katsuo-bushi", a Japanese traditional food processed from dried bonito possesses potent inhibitory activity against angiotensin I-converting enzyme (ACE). The present authors isolated eight kinds of ACE-inhibitory peptides from it. Of these isolated peptides, LKPNM ($IC_{50} = 2.4\ \mu M$) was found to be hydrolyzed by ACE to produce LKP ($IC_{50} = 0.32\ \mu M$) with 8-fold higher ACE-inhibitory activity relative to the parent peptide or LKPNM, suggesting that LKPNM can be regarded as a prodrug-type ACE-inhibitory peptide. For assessment of relative antihypertensive activities of LKPNM and LKP to that of captopril, they were orally administered to SHR rats to monitor time-course changes of blood pressures, whereby it was evidenced that both LKPNM and captopril showed maximal decrease of blood pressure 4 h after oral administration and their efficacies lasted until 6 h post-administration. In sharp contrast, however, maximal reduction of blood pressure occurred as early as 2 h after administration of LKP. Minimum effective doses of LKPNM, LKP and captopril were 8, 2.25 and 1.25 mg/kg, respectively. When compared on molar basis, antihypertensive activities of LKPNM and LKP accounted for 66% and 91% relative to that of captopril, respectively, whereas in vitro ACE-inhibitory activities of LKPNM and LKP were no more than 0.92% and 7.73% compared with that of captopril ($IC_{50} = 0.022\ \mu M$). It is of interest to note that both of these peptides exert remarkably higher antihypertensive activities in vivo despite weaker in vitro ACE-inhibitory effects, which was ascertained by using captopril as the reference drug.

Keywords: Angiotensin I-converting enzyme inhibitor; Antihypertensive peptides; Katsuo-bushi

1. Introduction

Angiotensin I-converting enzyme (ACE) converts angiotensin I to angiotensin II known to be a strong vasopressor, besides inactivating bradykinin conducive to lowering blood pressure (Ondetti et al., 1977). Eventually, it is well known that ACE inhibitors exhibit antihypertensive activity in spontaneously hypertensive rats (SHR) or hypertensive patients (Case et al., 1978). Recently, attention has been focused on various ACE-inhibitory peptides derived from casein (Maruyama et al., 1985, 1987a,b), fish muscle (Kohama et al., 1988; Suetuna and Osajima, 1989), and other proteins (Oshima et al., 1979; Maruyama et al., 1989). Previously, we found the thermolysin-digest of dried bonito to posses potent ACE inhibitory activity, culminating in our

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isolating eight kinds of peptides from it (Yokoyama et al., 1992). Out of these peptides, LKPNM showed long-lasting and dose-dependent antihypertensive activity after oral administration in SHR rats. The present report deals with unique profiles of LKPNM compared with those of captopril, a commercially available hypotensive agent. Antihypertensive activities of the thermolysin-digest of dried bonito containing LKPNM were also examined in SHR rats and hypertensive subjects.

2. Materials and methods

2.1. Materials

Rabbit lung acetone powder and captopril were purchased from Sigma while hippuryl–histidyl–leucine (HHL) was purchased from Peptide Institute. Peptides were synthesized according to *t*-Boc method (Biosearch SAM TWO).

2.2. Animals and hypertensive and borderline hypertensive subjects

SHR rats at 16 to 25 weeks of age, weighing 200 to 350 g were employed.

In a clinical study, enrolled were 30 subjects comprising hypertensive and borderline hypertensive subjects, whose blood pressures exceeded the standard of The Fifth Report of the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure; JNC-V (SBP > 130 mm Hg and DBP > 85 mm Hg).

2.3. Measurement of ACE-inhibitory activity

ACE inhibitory activity was determined by the method reported by Cushman and Cheung with minor modification by Yamamoto et al. (1989) and expressed by IC_{50}. Stability of the peptides against ACE was investigated following preincubation of the peptides with 28.8 mU of ACE at 37°C for 3 h.

2.4. Determination of blood pressure

After intravenous administration of peptides and captopril, their antihypertensive activities were determined as follows: SHR rats were anesthetized with α-D-chrolarose (40 mg/kg i.v.). Peptides and captopril, respectively dissolved in saline were injected into femoral vein using a catheter, followed by determination of carotid artery blood pressure with a pressure transducer (Spectramed Medical Products). On the other hand, following oral administration of peptides and captopril dissolved in saline via a gastric metal zonde, antihypertensive activities were also measured by the tail cuff method using UR-5000 (Ueda Seisakusho).

In a clinical study, blood pressures and heart rates in the enrolled subjects were determined by sphygmomanometer.

3. Results and discussion

3.1. Factors affecting antihypertensive activity after oral administration

For comparative purposes, we isolated ACE-inhibitory peptides from the thermolysin-digest of dried bonito, chicken muscle and the peptic digest of ovalbumin. Although these peptides from different sources had apparent ACE-inhibitory activities, some of them failed to show their antihypertensive activities after oral administration in SHR rats (Table 1). We noticed that the substrates for ACE also showed apparent ACE-inhibitory activities in the assay used for the screening and classified the ACE-inhibitory peptides into three groups as follows, based on the fates observed after preincubation with ACE.

(1) Inhibitor type: IC_{50} values of peptides are not affected despite of preincubation with ACE. As a natural consequence, these peptides still possess antihypertensive activities even after oral administration in SHR.

(2) Substrate type: Preincubation with ACE is associated with elevation of IC_{50} values of peptides because ACE degradates peptides of this type as its substrates.

(3) Prodrug-type inhibitor: Once incubated with ACE, these peptides are converted from substrates by hydrolysis to their true inhibitors indicative of smaller IC_{50} values against ACE relative to those of the parent peptides. These peptides are characterized to exert long-lasting antihypertensive activities after oral administration in SHR.

Table 1
IC_{50} for ACE and antihypertensive activities of peptides

Peptides	Origin	IC_{50} (μM)		Δ Blood pressure in SHR rats by p.o.[a] (max. Δ mm Hg)	
		− Preincubation	+ Preincubation		
Substrate type					
FKGRYYP	Chicken	5.8	34	0	
FFGRCVSP	OVA	0.4	4.6	0	
ERKIKVYL	OVA	1.2	6.0	0	
Inhibitor type					
IY	Bonito	2.31	1.9	−19	2 h
LW	OVA	6.8	6.6	−22	2 h
IKW	Chicken	0.21	0.18	−17	4 h
IKP	Bonito	6.9	3.4	−20	6 h
LKP	Bonito	0.32	0.32	−18	4 h
IWH	Bonito	3.5	3.5	−30	4 h
Prodrug type					
LKPNM	Bonito	2.4	0.76	−23	6 h
IWHHT	Bonito	5.8	3.5	−26	6 h

[a] Decrease of systolic blood pressure in SHR (60 mg/kg, p.o.).

Our positioning of LKPNM (IC_{50} = 2.4 μM) as a typical prodrug-type inhibitor peptide derived from the thermolysin-digest of dried bonito can be sustained, given the findings that LKP (IC_{50} = 0.32 μM) is obtained by hydrolysis of LKPNM with ACE, besides exerting 8-fold higher activities than those of the parent LKPNM.

3.2. Antihypertensive activities of LKPNM, LKP and captopril

3.2.1. After intravenous administration

Antihypertensive activities of LKPNM, LKP and captopril were examined following intravenous administration in SHR rats. Blood pressure was lowered by 30 mm Hg after intravenous injection of LKPNM at a dose of 100 μg/kg while LKP at a dose of 30 μg/kg, the active form of LKPNM reduced blood pressure by 50 mm Hg, suggesting more effective antihypertensive activity of LKP relative to LKPNM, in a dose-dependent manner in both cases. Reduction of systolic blood pressure by 50 mm Hg required 94.3 μg/kg of LKPNM and 30.7 μg/kg of LKP whereas the equipotent dose of captopril was 3.7 μg/kg. In summary, antihypertensive potencies of LKPNM and LKP after intravenous injection accounted for only 11.0% and 19.8% compared to that of captopril on molar basis (Table 2).

3.2.2. After oral administration

Antihypertensive activities of LKPNM and LKP were investigated following oral administration in

Table 2
IC_{50} for ACE and antihypertensive activities in SHR rats

Sample	ACE inhibition		Antihypertensive activity			
	IC_{50} (μM)	Relative Activity	i.v.[a] (μg/kg)	Relative activity (molar)	p.o.[b] (mg/kg)	Relative activity (molar)
Captopril	0.022	100	3.7	100	2.5	100
LKPNM	2.4	0.92	94.3	11.0	10.5	66.5
LKP	0.3	7.3	30.7	19.8	4.2	90.6

[a] Amount of samples to decrease SBP by 50 mm Hg.
[b] Amount of samples to decrease SBP by 10 mm Hg.

SHR rats. As shown in Fig. 1a, LKPNM lowered systolic blood pressure dose-dependently after oral administration, with its minimum effective dose being 8 mg/kg. Maximum reduction of blood pressure by LKPNM occurred 4 h after oral administration and such efficacy persisted even after 6 h.

The same was true in reducing systolic blood pressure after oral administration of LKP, with its minimum effective dose being 2.25 mg/kg. Maximum decrease in blood pressure by LKP was observed 2 h after oral administration, and the blood pressure was reverted to the basal level within 6 h post-administration (Fig. 1b). Viewed together, it is evident that LKPNM exerts long-lasting antihypertensive activity compared with LKP. It is conceivable that such different profiles of these peptides are attributable to the time required for adsorption of the longer peptide and also the time lag for enzymatic conversion of LKPNM into LKP by ACE in vivo.

Captopril lowered systolic blood pressure after oral administration and its minimum effective dose was 1.25 mg/kg (Fig. 1c). Maximum decrease in blood pressure by captopril was evidenced 4 h after oral administration. Reduction of systolic blood pressure by 10 mmHg after oral administration of LKPNM, LKP and captopril required 10.5 mg/kg, 4.2 mg/kg and 2.5 mg/kg, respectively.

Table 2 summarizes in vitro ACE-inhibitory activities and in vivo antihypertensive activities of LKPNM, LKP and captopril on molar basis in SHR rats. ACE-inhibitory activities of LKPNM and LKP were as low as 0.92% and 7.3% relative to that of captopril (IC_{50} = 0.022 μM). In in vivo antihypertensive assessments, the potencies of LKPNM and LKP after intravenous administration accounted for only 11.0% and 19.8% compared with that of captopril whereas the counterparts after oral administration were remarkably elevated to 66.5% and 90.6% compared with that of captopril, respectively. Interestingly, IC_{50} values of LKPNM and LKP were much higher than that of captopril; however, these peptides showed antihypertensive activities comparable to captopril after oral administration in SHR rats. All of these findings indicate that ACE inhibitors derived from food protein possess higher in vivo activities than the efficacy levels extrapolated from in vitro activities. Considering lack of both vasodilating activity and inhibitory activity for neutral endopeptidase (data are not shown) of LKPNM and LKP, we might attribute these unique properties to higher affinity of these peptides to tissues and slower elimination than did captopril, a synthetic compound.

3.3. Antihypertensive activity of thermolysin-digest of dried bonito in SHR rats and hypertensive subjects

3.3.1. After long-term administration in SHR rats

After long-term administration for 7 weeks, the thermolysin-digest of dried bonito suppressed elevation of systolic blood pressure in SHR rats dose-de-

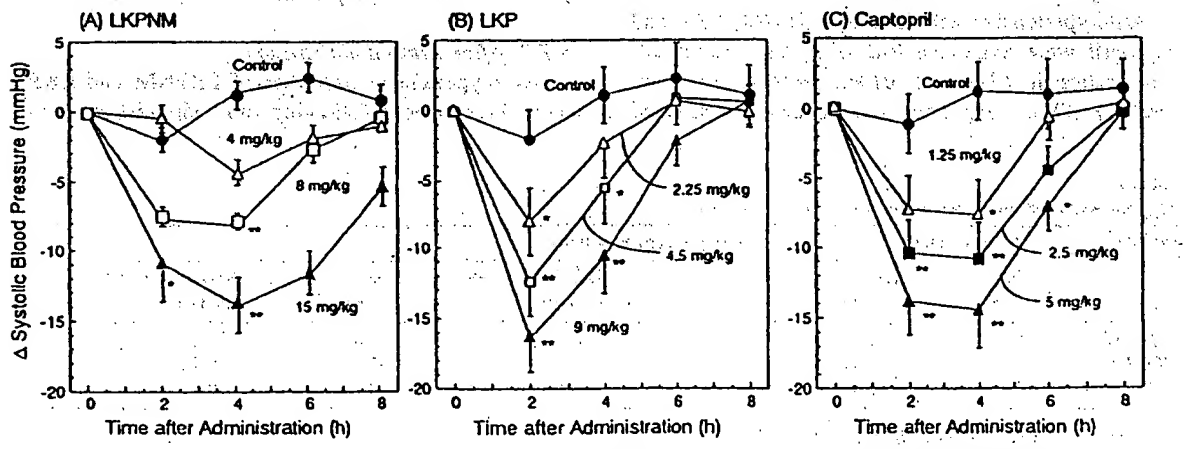

Fig. 1. Antihypertensive activity of LKPNM (A), LKP (B) and captopril (C) after oral administration in SHR rats. Changes of systolic blood pressure from zero time were expressed with mean ± S.E. *, ** indicate significance against control (* $P < 0.05$, ** $P < 0.01$; *t*-test, 8 *d.f.*).

pendently. Following 5-week administration, elevation of systolic blood pressure was significantly inhibited by the thermolysin-digest at a dose of 15 mg/kg/day while the difference from those in the control group reached 23 mm Hg after 7 weeks. In the meantime, after a single oral administration of the thermolysin-digest at a dose of 50 mg/kg, systolic blood pressure was significantly lowered in SHR rats, with maximal reduction by 22 mm Hg ($P < 0.01$) in systolic blood pressure being observed 6 h after administration. Given these findings obtained either in long-term dosing or in a single oral administration, long-term ingestion lowered the minimal effective dose of the thermolysin-digest to 1/33 compared with that after single oral administration. The thermolysin-digest contains LKPNM, the prodrug-type ACE-inhibitory peptide characteristic of demonstrating long-lasting antihypertensive activity; therefore, it is conceivable that this peptide might play a role in reducing the minimum dose as evidenced in the long-term administration study.

3.3.2. Antihypertensive activity of thermolysin-digest of dried bonito in hypertensive and borderline hypertensive subjects

After 4-week observation period, 30 subjects were assigned to two groups to receive digest either by ingestion (3 g/day) or by non-ingestion for 8 weeks, and then crossed over for another 8 weeks. Ingestion of the digest lowered blood pressure in the first ingestion group by 12.7 mm Hg, with the effective ratio being 60.0%. The digest also reduced blood pressure in the latter ingestion group by 12.4 mm Hg, with the effective ratio accounting for 66.6%.

4. Conclusion

LKPNM is the prodrug-type ACE inhibitory peptide derived from the thermolysin-digest of dried bonito. Following hydrolysis of LKPNM into LKP by ACE, an 8-fold augmentation in ACE-inhibitory activity was observed. LKPNM showed almost the equipotent antihypertensive activities to captopril on a molar basis after single oral administration in SHR rats, besides exerting long-lasting antihypertensive activity comparable to captopril. In a small scale clinical study, the thermolysin-digest of dried bonito evidenced long-lasting antihypertensive activity after oral administration in hypertensive and borderline hypertensive subjects. Incidentally, this digest called "Katsuo-bushi oligopeptide" has been officially approved as Foods for Specified Health Use by the Ministry of Health and Welfare in Japan.

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